Regulation of Ace2-dependent genes requires components of the PBF complex in *Schizosaccharomyces pombe*

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ABSTRACT

The division cycle of unicellular yeasts is completed with the activation of a cell separation program that results in the dissolution of the septum assembled during cytokinesis between the two daughter cells, allowing them to become independent entities. Expression of the *eng1*$^+$ and *agn1*$^+$ genes, encoding the hydrolytic enzymes responsible for septum degradation, is activated at the end of each cell cycle by the transcription factor Ace2. Periodic *ace2*$^+$ expression is regulated by the transcriptional complex PBF (PCB Binding Factor), composed of the forkhead-like proteins Sep1 and Fkh2 and the MADS box-like protein Mbx1. In this report, we show that Ace2-dependent genes contain several combinations of motifs for Ace2 and PBF binding in their promoters. Thus, Ace2, Fkh2 and Sep1 were found to bind *in vivo* to the *eng1*$^+$ promoter. Ace2 binding was coincident with maximum level of *eng1*$^+$ expression, whereas Fkh2 binding was maximal when mRNA levels were low, supporting the notion that they play opposing roles. In addition, we found that the expression of *eng1*$^+$ and *agn1*$^+$ was differentially affected by mutations in PBF components. Interestingly, *agn1*$^+$ was a major target of Mbx1, since its ectopic expression resulted in the suppression of Mbx1 deletion phenotypes. Our results reveal a complex regulation system through which the transcription factors Ace2, Fkh2, Sep1 and Mbx1 in combination control the expression of the genes involved in separation at the end of the cell division cycle.

INTRODUCTION

The proliferation of all organisms depends on the process of cell division, in which a single cell duplicates and divides to produce two genetically identical daughter cells. This requires a strict temporal and spatial coordination of different processes and mechanical systems that contribute to ensuring successful cell division. *Schizosaccharomyces pombe* provides an excellent model to study the different control mechanisms that regulate the progression between the stages of the cell division cycle. One of these mechanisms is the transcriptional control of gene expression (for reviews, see refs.[1,2)]. The regulation of gene transcription ensures that proteins required at particular cell cycle moment are only produced when they are needed, and it is a universal mechanism used by proliferating cells for orderly cell cycle progression. In *S. pombe,* genome-wide gene expression analyses have identified four major waves of transcription through the mitotic cycle whose periodic patterns coincide with the main stages of the cell cycle.[3-5] The molecular mechanisms by which the expression of the different groups occurs have been partially deciphered and found to involve *cis*-acting DNA motifs present in the co-regulated promoters to which a *trans*-acting transcription factor complex specific for each group of genes binds.[2] The different combinations of these elements for each group results in coordinated gene expression at different cell cycle times.

For most cells, division of the nucleus (mitosis) precedes division of the cytoplasm (cytokinesis), both processes being closely interlinked in order to ensure the distribution of the genetic material among the resulting daughter cells. Cytokinesis requires the assembly and constriction of an actomyosin ring that provides the force necessary for cytoplasm partition, a process that is coordinated with the synthesis of the new membrane that separates the cells. In organisms with a cell wall, ring contraction is also coupled to the synthesis of a trilaminar structure, called the septum, between the two daughter cells; this is fundamental for cell integrity to be ensured during cytokinesis. In unicellular organisms, cytokinesis is followed by the controlled degradation of the central layer of the newly synthesized septum, known as the primary septum, allowing the two daughter cells to separate from each other physically (cell separation) and become independent entities.[6-8] Cytokinesis and cell separation therefore need to be highly coordinated in time and they must be spatially regulated, since they are opposite processes (septum formation and septum degradation) that must occur within minutes. To avoid cell lysis during cytokinesis, dissolution of the primary septum does not start until its synthesis has been completed, and the enzymes involved in its dissolution must be targeted to the region of the cell where the septum has been assembled.

Cell separation in *S. pombe* is controlled transcriptionally by the Sep1-Ace2 cascade.[4,9] Sep1 is a protein of the conserved forkhead family[10,11] that activates the expression of the

first of two transcriptional waves that occur at the end of the cell cycle, during the M-G1 transition, which is the moment when cytokinesis and cell separation occur.[3-5,12] This cluster of co-expressed genes includes genes such as *plo1*$^+$, *cdc15*$^+$, *ppb1*$^+$, *slp1*$^+$, *fin1*$^+$ or *sid2*$^+$, whose products are necessary for the completion of mitosis and cytokinesis. Another target of Sep1 is *ace2*$^+$, which encodes a C2H2 zinc-finger transcription factor that in turn activates a second wave of gene expression, with maximum expression at the end of mitosis.[4,9,13] *ace2*$^+$ periodicity has been shown to be conserved in multiple organisms.[14] Among the genes regulated by Ace2 in *S. pombe* are those that encode the main enzymes responsible for the dissolution of the cell division septum, such as the β-glucanase Eng1, which degrades the primary division septum;[15] the α-glucanase Agn1, which hydrolyses the old cell wall surrounding the septum [16,17] and Adg proteins of unknown function that are also necessary for cell separation.[9] Thus, a major function of the Sep1-Ace2 transcriptional pathway *in S. pombe* is to periodically trigger the expression of the genes required for completion of cytokinesis and cell separation at the end of the cell cycle. At the same time, the existence of two different transcription factors allows a delay in the expression of cell separation genes over those required for the completion of cytokinesis. Mutants lacking Sep1 or Ace2 have similar defects in septum degradation, producing a typical phenotype of chains of unseparated cells.[9,15,18] A more modest cell separation defect can be also found in mutants lacking the Eng1, Agn1 or Adg proteins.[9,15-17]

Sep1 is part of a transcriptional complex known as PBF (Pombe cell cycle box Binding Factor), which includes at least two other transcription factors, namely the forkhead-like protein Fkh2 and the MADS box-like protein Mbx1.[12,19-22] Although forkhead proteins are generally believed to be “actipressors” (they can both activate and repress gene expression),[23] the two forkhead-like proteins of the PBF complex in *S. pombe*, Sep1 and Fkh2, are thought to play opposing roles in regulating mitotic transcription.[19,24] The activator role of Sep1 is supported by the observation that the deletion of *sep1*$^+$ causes reduced transcription in mitosis, whereas its overexpression results in the induction of the target genes. Furthermore, maximum levels of Sep1 binding to mitotic gene promoters coincide with maximum transcription levels.[19] In contrast, Fkh2 binding is observed when gene expression is waning and the absence of this transcription factor protein elicits constitutive expression levels along the cell cycle.[19, 20] Sep1 is required for Fkh2 activity, since it has been observed that the deletion of the *sep1*$^+$ gene suppresses the lethal phenotype observed when *fkh2*$^+$ is over-expressed; the two proteins have also been shown to interact with each other in cells.[19, 24] The third factor, Mbx1, is not necessary for the periodicity of genes transcribed during M phase.[19] How PBF activity is regulated and integrated with the cell cycle is not yet fully understood. Recent studies have revealed that Mbx1 and Fkh2 are controlled through temporary phosphorylation by the kinases Plo1 (Polo kinase) and the

cyclin-dependent kinase Cdk8, while dephosphorylation is performed by the Clp1 phosphatase.[24-26] In fact, Sep1 is probably a phosphoprotein controlled by Cdk1, and it is also a substrate of the Clp1 phosphatase.[27,28] It has also been shown that Sak1, a transcription factor of the RFX family, activates the expression of mitotic genes in collaboration with Fhk2. [29]

Promoters of the genes thought to be under the control of PBF (including *ace2*$^+$) are significantly enriched in forkhead-binding motifs.[3,4] These *cis*-acting DNA regulatory elements are conserved from humans to budding yeast. Unlike budding yeast, *S. pombe* forkhead-binding sites do not seem to be accompanied by consensus motifs for MADS-box-like protein binding.[30,31] Instead, they are often accompanied by DNA-binding sites known as PCB (Pombe cell-Cycle Box; consensus GNAACG/A).[12]

Less is known, however, about the regulation of the genes under the control of Ace2. The promoters of the genes under the control of this transcription factor are enriched for the hexanucleotide CCAGCC.[3,4] This sequence has also been described as the consensus binding site for the Ace2 ortholog in *S. cerevisiae* and it is necessary for proper transcriptional regulation.[32,33]

In this report, we show that Ace2 consensus motifs are required for Ace2 binding to the promoter region of their regulated genes. In addition, this recruitment occurs during periods of maximum expression and is decreased when another transcription factor, Fkh2, is detected. We also demonstrate that the PBF components Sep1, Fkh2 and Mbx1 contribute to the regulation of Ace2-dependent genes, but that there are significant differences between individual genes. Thus, Sep1 appears to be involved in activating *eng1*$^+$ expression whereas Mbx1, which has not been thought to be relevant for mitotic gene control in *S. pombe*, is required for *agn1*$^+$ expression. Our findings suggest a complex array of control mechanisms aimed at ensuring the correct timing of gene expression at the end of the cell cycle to complete cytokinesis, septation and separation.

RESULTS

CCAGCC-binding sites are required for maximum expression mediated by Ace2

Analysis of the promoter region of the *eng1*$^+$ gene revealed the presence of two candidate motifs that matched the predicted consensus Ace2-binding sequence (CCAGCC), located at -413 and -478 bp upstream of the ATG. To test whether these putative binding sites were important for the expression of Ace2-dependent genes, we constructed three versions of the *eng1*$^+$ promoter region (from -600 to -1) in which either one or both copies of these sites were deleted. The three mutated promoters (referred to as *eng1*-Δ*1*, *eng1*-Δ*2*, and *eng1*-Δ1Δ*2*) were cloned upstream of the *lacZ* reporter gene on a plasmid and introduced into a wild-type strain. As controls, the wild-type and *ace2*Δ

strains carrying a plasmid with the wild-type version of the *eng1*$^+$ promoter (*eng1*-wt) were used. Analyses of β-galactosidase activity in asynchronously growing cells revealed reduced levels of enzymatic activity in cells carrying either the *eng1*-Δ*1* or the *eng1*-Δ*2* single-mutated promoters (around 40% and 30% of that of the wild-type, respectively) (Figure 1A). This effect was additive, since β-galactosidase activity was further reduced to around 10% of the wild-type activity when the two putative Ace2-binding sites were deleted (*eng1*-Δ1Δ2). As expected, *lacZ* expression was also reduced in cells carrying the wild-type *eng1*$^+$ promoter but lacking the transcriptional activator Ace2 (*ace2*Δ strain), although this strain still exhibited around 20% of the β-galactosidase activity found in the wild-type strain (Figure 1A). Thus, importantly, these results indicate that the two copies of the CCAGCC sequence present at the *eng1*$^+$ promoter are important and necessary to achieve maximum expression of its coding region.

We next performed quantitative chromatin immunoprecipitation analyses (ChIP-qPCR) to determine the binding capacity of Ace2 to the CCAGCC motifs *in vivo.* To this end, the double-mutated version of the *eng1*$^+$ promoter was integrated into its own chromosomal locus by site-directed mutagenesis (*eng1*-Δ1Δ2 allele) in a strain carrying Ace2 tagged with the HA epitope at its C-terminus. Both the wild-type and the *eng1*-Δ1Δ2 mutant strains were used to test Ace2 binding to the *eng1*$^+$ promoter. As a control, we used another of the Ace2-dependent genes, *agn1*$^+$. The results of ChIP-qPCR in asynchronous cultures showed that Ace2 was recruited to the wild-type promoter regions of *eng1*$^+$ and *agn1*$^+$, and that binding was specifically reduced in the strain carrying the *eng1*-Δ1Δ2 allele (Figure 1B). This result meant that the CCAGCC motifs are also binding sites in *S. pombe* for Ace2 *in vivo*. In agreement with these data, *eng1*$^+$ expression was reduced in cells lacking Ace2 or the two Ace2-binding sites at the *eng1*$^+$ promoter (Figure 1C). Taken together, these results demonstrate that in fission yeast, as in *S. cerevisiae*, Ace2 activates the expression of its target genes directly through its binding to the CCAGCC consensus sequence motifs.

Ace2 binding to DNA is cell cycle-regulated

For a more detailed analysis of Ace2 and its function as a transcriptional activator, we synchronized cells using the *cdc25-22* mutant and monitored both the expression levels of *eng1*$^+$ and *agn1*$^+$ and the recruitment of Ace2 to their promoters through the cell cycle. As previously described,[9] the expression of *eng1*$^+$ and *agn1*$^+$ occurred in a periodic manner, with a maximum at anaphase (Figure 1D). Interestingly, Ace2 was also found to bind the two promoters in a cyclic manner, with binding peaks coincident with the maximum mRNA levels of both genes (Figure 1E). Therefore, cell cycle-dependent recruitment of Ace2 to the promoters might be responsible for the periodic expression of genes under the control of this transcription factor. We also noted that Ace2 binding to the *eng1*$^+$ promoter occurred

earlier than to *agn1*$^{+}$ (Figure 1E); this was consistent with an earlier induction of *eng1*$^{+}$ expression seen for this gene in synchronized cultures (Figure 1D).

The promoters of Ace2-target genes contain additional regulatory elements

Examination of the promoters of genes under the control of Ace2 allowed us to identify other possible *cis*-acting regulatory elements. For this analysis, we used the upstream regions of 23 genes reported to be regulated by Ace2 by microarray experiments,[4,9] and the Regulatory Sequence Analysis Tools program, RSAT.[34] As expected, most of the promoters (19 out of 23) contained one or more copies of the Ace2 consensus-binding site. In fact, 16 promoters contained at least two CCAGCC motifs (Figure 2). Furthermore, we found that most of these Ace2 sites were located in nucleosome-depleted regions (NDR) close to the transcription starting sites.[35] The localization of transcription factor binding sites in NDR regions is believed to enhance transcription factor attachment and facilitate subsequent transcription.[36] In *S. cerevisiae,* it has been shown that NDRs are important for maintaining the periodic expression of cell cycle-regulated genes.[37] In addition to Ace2-binding sites, the RSAT program also detected the consensus motif TGTTTA, which has been reported to be a binding site for forkhead transcription factors.[30] This motif was over-represented in the promoters analyzed and was frequently accompanied by PCB sites. For example, in the *eng1*$^{+}$ promoter two forkhead and one PCB site were present within the NDR. In striking contrast, *agn1*$^{+}$, the other glucanase involved in septum degradation, together with *eng1*$^{+}$, was one of the few genes with no consensus forkhead or PCB motifs, suggesting that these two glucanases might be subject to a different type of regulation.

The presence of potential forkhead- and/or PCB-binding sites in many of the promoters regulated by Ace2 suggested that the PBF complex might also bind to the promoters of Ace2-dependent genes. As a first approach to investigate this possibility, we carried out electrophoretic mobility shift assays (EMSA) using a DNA fragment from the *eng1*$^{+}$ promoter region (-578 to -335) that contained two Ace2 sites, two forkhead sites, and a single PCB site as a probe. For this experiment, protein extracts from single-, double- and triple-deletion mutants lacking the regulators Ace2, Fkh2 and Mbx1 were prepared and examined for their ability to bind to labeled probe DNA in comparison with protein extracts from a wild-type strain. Incubation of the probe with a wild-type extract uncovered the presence of a retarded complex, indicating the association *in vitro* of a protein with the fragment of the *eng1*$^{+}$ promoter (Figure 3A). Interestingly, different degrees of retardation of the probe were observed when extracts from single (*ace2*Δ*, fkh2*Δ or *mbx1*Δ) or double mutants (*ace2*Δ *fkh2*Δ*, ace2*Δ *mbx1*Δ*,* or *fkh2*Δ *mbx1*Δ*)* were tested, supporting the idea that Ace2, along with Fkh2 and Mbx1, might contribute to the regulation of Ace2-target

genes through binding to its promoter. Significantly, no mobility shift was observed upon incubation of the probe with a protein extract from the triple mutant lacking Ace2, Fkh2 and Mbx1 (*ace2*Δ *fkh2*Δ *mbx1*Δ), suggesting that no other proteins can bind to the *eng1*$^+$ promoter region, at least *in vitro*. Thus, these EMSA experiments suggest that the *eng1*$^+$ promoter fragment has the ability to bind Ace2, Fkh2 and Mbx1 *in vitro*.

The forkhead proteins Sep1 and Fkh2 bind to the promoters of Ace2-target genes *in vivo*

We next investigated whether the transcription factors that make up the PBF complex were recruited to the promoters of Ace2-target genes (*eng1*$^+$, *agn1*$^+$, *adg1*$^+$, *adg2*$^+$ and *adg3*$^+$) *in vivo*. To this end, quantitative ChIP experiments using asynchronous cultures of strains containing C-terminally tagged versions of these three transcription factors (Sep1-myc, Fkh2-HA, and Mbx1-HA) were used. As a control, binding to *slp1*$^+$ and *fkh2*$^+$ promoters was also analyzed, since it is known that they are regulated by PBF.[19] In addition, recruitment of Ace2 was also measured for comparative purposes. As expected, Ace2 was present in the promoters of all five genes tested, although between them differences in binding were observed that correlated with the number of Ace2 binding sites present in their sequences. Thus, the promoters with at least two sites, such as *eng1*$^+$, *agn1*$^+$ and *adg2*$^+$, contained the highest amount of Ace2, while *adg1*$^+$ and *adg3*$^+$, with only one Ace2 binding site, showed a less marked recruitment of this protein (Figures 2 and 3B). Importantly, the forkhead-like transcription factor Fkh2 was detected in the promoters of *eng1*$^+$ (with values comparable to those of the positive control, *fkh2*$^+$) and *adg2*$^+$. In contrast, limited binding was observed in the promoters of *agn1*$^+$, *adg1*$^+$ and *adg3*$^+$. Again, the reduced binding to these genes correlated with a lower number of forkhead/PCB sites present in their promoters (Figures 2 and 3B). For the transcription factor Sep1, we observed recruitment of this protein to the *eng1*$^+$ and *adg2*$^+$ promoters, but binding was lower than that detected for Fkh2 (Figure 3B).

We were unable to detect Mbx1 binding to any of the promoters tested (data not shown), similar to the results reported for other PBF-regulated genes.[24] In contrast, the Polo kinase Plo1 interacts with Mbx1 throughout the cell cycle and is detected on Mbx1-target gene promoters. Thus, we examined Plo1 association with the promoters of Ace2-dependent genes, but no binding was detected (data not shown).

Since the strongest binding detected by ChIP corresponded to the *eng1*$^+$ promoter and since this gene is adjacent and divergently transcribed to *slp1*$^+$, which is also regulated by PBF,[19] it is possible that the signal observed for *eng1*$^+$ could have been due to the binding of PBF to the *slp1*$^+$ promoter. To rule out this hypothesis, seven different oligonucleotide pairs annealing at different regions of the *slp1*$^+$-*eng1*$^+$ intergenic region were designed and

used to measure the abundance of each fragment in immunoprecipitates of a strain carrying Fkh2-HA (a to g in Figure 3C). As a control, recruitment of Ace2 to the same regions was examined in an Ace2-HA strain. This analysis revealed that Ace2 ChIP-enrichment binding was restricted to the promoter region upstream of *eng1*$^+$ (400-500 nt upstream of the ATG; Figure 3C). No Ace2 binding to the coding region of *eng1*$^+$ or to the *slp1*$^+$ promoter was observed. In contrast, Fkh2 showed two different binding peaks upstream of *eng1*$^+$ and *slp1*$^+$ (c and f, Figure 3C) that were separated by a region where no recruitment was observed (e, Figure 3C), corresponding to a non-coding RNA region (*SPNCRNA.163*). This result indicated that Fkh2 binding to the *eng1*$^+$ and *slp1*$^+$ promoters was independent in both cases. Therefore, the ChIP analyses support the idea that the PBF transcriptional complex might be involved in the regulation of the expression of Ace2-dependent genes, at least those containing forkhead and/or PCB sites in the promoter, such as *eng1*$^+$.

eng1$^+$ and _agn1_$^+$ are subject to different transcriptional regulation

The ChIP results suggested that different transcriptional regulators bound to the promoter regions of Ace2-dependent genes. To test whether they performed a function in the control of the expression of this group of genes, the effect of deleting Fkh2, Sep1, and Mbx1 on mRNA expression of Ace2-target genes was analyzed, focusing the study on *eng1*$^+$ and *agn1*$^+$ as representatives of genes with or without forkhead/PCB-binding sites respectively. Since it has been described that *ace2*$^+$ is itself a PBF-regulated gene,[19,20] *ace2*$^+$ transcription levels were also monitored. Quantitative mRNA measurements in asynchronously growing cells showed that *eng1*$^+$ and *agn1*$^+$ expression was decreased in strains lacking Sep1 or Ace2 (Figure 4A). This is consistent with previously published results showing that Ace2 activates the transcription of genes involved in cell separation, such as *eng1*$^+$ and *agn1*$^+$, and that Sep1 indirectly controls the expression of Ace2-target genes through the activation of *ace2*$^+$ expression.[9] Strikingly, however, we found that the expression of *eng1*$^+$, but not *agn1*$^+$, was even lower in *sep1*Δ cells than in the *ace2*Δ mutant. This reduction in *eng1*$^+$ expression in *sep1*Δ cells occurred when *ace2*$^+$ mRNA levels were at 20% relative to the wild-type, suggesting that Sep1 might also directly influence *eng1*$^+$ expression.

The forkhead-like protein Fkh2 has been reported to act as a repressor of the expression of *ace2*$^+$ and other Sep1-dependent genes.[19,20] In agreement with this function, asynchronous cells lacking Fkh2 displayed up-regulated expression levels of *ace2*$^+$ (Figure 4A). Similarly, the expression of both *eng1*$^+$ and *agn1*$^+$ was increased (Figure 4A).

It has been suggested that the MADS box protein Mbx1 is not required for periodic gene regulation, but that instead it plays a role in controlling the amplitude of expression, since

this is reduced in *mbx1*Δ cells.[19] We noted that deletion of *mbx1*$^+$ reduced the expression of the two glucanase-encoding genes. *agn1*$^+$ expression was reduced in the *mbx1*Δ mutant to nearly the level observed in the *ace2*Δ and *sep1*Δ strains; this was surprising because the *agn1*$^+$ promoter does not contain any consensus PBF binding sites (Figure 4A). *ace2*$^+$ mRNA abundance in asynchronous cultures of the *mbx1*Δ strain was reduced to around 35% of the wild-type (Figure 4A). Nevertheless, this change in *ace2*$^+$ expression could not be the only explanation for the relatively low *agn1*$^+$ expression in the *mbx1*Δ strain. Furthermore, deletion of *fkh2*$^+$ suppressed the decreased expression of *ace2*$^+$ in a *mbx1*Δ background, but *agn1*$^+$ mRNA levels were still low in the double mutant *mbx1*Δ *fkh2*Δ (Figure 4A).

Since the deletion of *fkh2*$^+$ and *mbx1*$^+$ changed the expression of *ace2*$^+$,[19] the effect of Fkh2 and Mbx1 on Ace2-dependent genes could be indirect due to differences in the abundance of the Ace2 transcription factor in these mutant strains. Therefore, to define the possible direct role of the PBF transcriptional complex on Ace2-dependent genes, a new set of strains in which *ace2*$^+$ was cloned under the control of a PBF-independent promoter was constructed. To accomplish this, the thiamine-repressible *nmt1*$^+$ promoter was integrated into the genome by homologous recombination directly upstream of the *ace2*$^+$ ORF in the wild-type and in mutant strains lacking Fhk2, Sep1 or Mbx1. After the induction of *ace2*$^+$ transcription by growth in thiamine-free medium, the overall expression level of *eng1*$^+$ and *agn1*$^+$ was comparable to that of the wild-type strain (data not shown). In contrast, under non-inducing conditions (*ace2*$^+$ transcription off) the P*nmt1*$^+$*-ace2*$^+$ strain showed low expression levels of *eng1*$^+$ and *agn1*$^+$*,* similar to those observed in the *ace2*Δ mutant. When the mutant strains P*nmt1*$^+$*:ace2*$^+$ *sep1*Δ, P*nmt1*$^+$*:ace2*$^+$ *fkh2*Δ and P*nmt1*$^+$*:ace2*$^+$ *mbx1*Δ were grown under inducing and non-inducing conditions, different patterns of *eng1*$^+$ and *agn1*$^+$ expression were observed (Figure 4B). While *agn1*$^+$ mRNA levels were markedly reduced in the absence of Mbx1 (Figure 4B, column 3), *eng1*$^+$ was down-regulated in *sep1*Δ cells (Figure 4B, column 5). Furthermore, combining the *sep1*Δ mutation with the depletion of *ace2*$^+$ caused an additional reduction in *eng1*$^+$ expression (Figure 4B, column 6), indicating an additive effect of Sep1 and Ace2 in the regulation of *eng1*$^+$. Cells lacking Fkh2 showed a slight decrease in the expression of both *eng1*$^+$ and *agn1*$^+$ (Figure 4B, column 7). Taken together, these results indicate that the transcriptional regulation of the Ace2-dependent genes involves different factors in addition to Ace2, and that these factors may vary between genes.

Fkh2 binds to the *eng1*$^+$ promoter in a cell-cycle dependent manner

To further investigate the role of Fkh2 in the regulation of Ace2-dependent genes, we synchronized cells using the *cdc25-22* mutant and followed the kinetics of Fkh2 binding to

the promoters of *eng1*$^+$ and *agn1*$^+$ through the cell cycle. ChIP analyses revealed that Fkh2 binding to the *eng1*$^+$ promoter region was periodic during the cell division cycle (Figure 5A), although with a different pattern to that observed for Ace2 (Figure 1D). Fkh2 recruitment to the *eng1*$^+$ promoter increased after anaphase, when *eng1*$^+$ transcription decreased (Figure 5A). No detectable binding was observed at anaphase, when maximum *eng1*$^+$ expression occurs, consistent with Fkh2 being a transcriptional repressor. Similar binding profiles for Fkh2 have been detected in PCB-regulated promoters, where Fkh2 is also thought to play a role as a repressor. [24] In agreement with previous results in non-synchronized cells, no clear binding signals for Fkh2 were observed at the *agn1*$^+$ promoter, supporting the notion that different molecular mechanisms operate in the transcriptional regulation of *eng1*$^+$ and *agn1*$^+$.

To analyze the contribution of Fkh2 to the regulation of *eng1*$^+$ in more detail, we measured *eng1*$^+$ expression through the cell cycle in a P*nmt1*$^+$*:ace2*$^+$ *fkh2*Δ mutant in comparison with P*nmt1*$^+$*:ace2*$^+$ cells. For this experiment, cells were arrested in early mitosis using the cold-sensitive *nda3-KM311* mutant, since the *cdc25-22 fkh2*Δ P*nmt1*$^+$*:ace2*$^+$ mutant shows synthetic sick phenotypes, which precluded its use for analysis. Under these conditions, we observed that in thiamine-free medium the expression profile for *eng1*$^+$ was similar in both the P*nmt1*$^+$*:ace2*$^+$ and the P*nmt1*$^+$*:ace2*$^+$ *fkh2*Δ strains, with high mRNA levels in arrested cells (most of the cells were in metaphase), after which they decreased (Figure 5B). This result indicated that the observed Fkh2 recruitment to the *eng1*$^+$ promoter is unlikely to be the mechanism responsible for repressing its transcription. As could be expected, *agn1*$^+$ expression was also periodic in both strains, although the amplitude of the oscillation was lower in the strain lacking Fkh2. Additionally, this experiment allowed us to verify that *agn1*$^+$ expression was delayed by 20 min (most of the cells were in late anaphase) as compared to that of *eng1*$^+$.

We also examined the effect of point mutations in forkhead- and PCB-binding sites in the promoter of *eng1*$^+$. For this, we generated strains carrying point mutations in the three forkhead sites and in the PCB site of the *eng1*$^+$ promoter integrated at its chromosomal locus (P*eng1-4m* allele, indicated by asterisks in Figure 2). ChIP analyses revealed a reduction in the recruitment of Fkh2 and Sep1 to the *eng1*$^+$ promoter in P*eng1-4m* cells as compared to the wild-type promoter (Figure 5C). However, these mutations did not affect Ace2 recruitment (data not shown). In spite of the reduction of Fkh2 and Sep1 recruitment, RT-qPCR analyses using RNA isolated from synchronized cultures showed similar expression profiles of *eng1*$^+$ in cells carrying the mutated promoter or the wild-type promoter (Figure 5D). Additionally, we also examined whether Fkh2 binding to the *eng1*$^+$ promoter was dependent upon Ace2 sites. Thus, Fkh2 binding was measured in a strain carrying the two Ace2-binding sites at the *eng1*$^+$ promoter deleted (*eng1*-Δ1Δ2 allele, strain

YMAT84). The results of ChIP analyses showed that a similar Fkh2 recruitment had occurred in both the wild-type and the mutated promoter (data not shown). Overall, these results suggest that additional regulatory elements for forkhead transcription factor binding probably exist in the *eng1*$^+$ promoter, and that Ace2 and Fkh2 bind independently to different elements of the promoter.

Mbx1 is required for *agn1*$^+$ expression

The fact that Mbx1 had a specific effect on *agn1*$^+$ expression was unexpected for two reasons: first, the *agn1*$^+$ promoter had no consensus-binding sites for PBF, and second, Mbx1 has not been shown to be a critical transcription factor for gene expression until now. To obtain additional evidence that Mbx1 was controlling the expression of *agn1*$^+$, we monitored *agn1*$^+$ and *eng1*$^+$ expression during the cell cycle progression in *cdc25-22 mbx1*Δ cells. Similar to the results of the asynchronous cultures, *agn1*$^+$ expression was lost in the absence of Mbx1, whereas periodic *eng1*$^+$ transcription was unaffected (Figure 6A). Therefore, *agn1*$^+$ expression is dependent on Mbx1.

Since Agn1 is an α-1,3-glucanase required for the degradation of the cylinder of cell wall that surrounds the septum, known as the septum edging,[16,17] *agn1*Δ mutants have a typical V-shaped cell separation defect in which the two daughter cells remain attached by the remnants of cell wall on one side (Figure 6B).[9] It has been described that *mbx1*Δ mutants also have a cell separation defect,[19] and our results suggest that it could be attributed to the absence of Agn1. Therefore, we tested whether replacement of the *agn1*$^+$ promoter by a heterologous promoter to allow *agn1*$^+$ expression independent of Mbx1 could reverse the cell separation defect of *mbx1*Δ mutants. Quantification of the cell separation phenotype revealed that around 98% of the separating cells had a V-shaped morphology in *agn1*Δ mutants (Figure 6B). A similar percentage was observed in the *mbx1*Δ mutant and in *mbx1*Δ P*nmt1*$^+$*:agn1*$^+$ cells grown under repressing conditions. Interestingly, overexpression of *agn1*$^+$ largely corrected the phenotype of *mbx1*Δ cells, restoring a wild-type phenotype. Therefore, these results confirm the idea that transcriptional activation of *agn1*$^+$ by Mbx1 is essential for normal separation of mother and daughter cells after cytokinesis.

DISCUSSION

The regulation of cell cycle progression is of key importance for proper segregation of the genome and the generation of two identical cells. One way to ensure a correct order of cell cycle events is the control of specific proteins at certain phases of the cell cycle, and this can be achieved either by altering the stability of the proteins or through the regulation

of gene expression. The latter mechanism ensures the coordinated expression of groups of genes at particular moments of the cell cycle, when their products are required, and it is a widespread mechanism among eukaryotic cells.

In budding yeast, the consecutive waves of gene expression during the different stages of the cell cycle are functionally linked to one another by mechanisms in which one wave of gene expression contains the transcription factor that controls the transcription of the next wave of gene expression.[38-41] In fission yeast, transcriptome analyses have unveiled the existence of four main waves of gene expression, corresponding to several stages of the cell cycle,[3-5] although there is a low degree of linkage between them. To date, the only direct link between two consecutive cell cycle waves of transcription occurs at the end of mitosis, and it is the transcriptional cascade involving Sep1 and Ace2 transcription factors.[1,2] In this study, we examined the regulation of genes encoding proteins required for cell separation whose expression occurs periodically at the M-G1 interval, dependent on the transcription factor Ace2. We found that the regulation of this group of genes involved other transcription factors in addition to Ace2, such as the PBF components. Furthermore, the regulation of this group of genes showed different degrees of dependence on Sep1, Fkh2 and Mbx1.

In *S. cerevisiae*, the paralogs Swi5 and Ace2 regulate a wave of gene expression at M-G1 by binding to the ACCAGCN sequence present at the promoters of the target genes.[32,33,42,43] Transcriptome analyses in *S. pombe* have identified a group of 23 genes that are expressed at the M-G1 transition that require the transcription factor Ace2.[3-5,9] Most of them contain one or more copies of the consensus sequence ACCAGCC in their promoters. *eng1*$^+$ contains two copies of this sequence, which are necessary for the expression of the gene and for Ace2 binding, indicating that in *S. pombe* Ace2 has the same binding specificity as in *S. cerevisiae*. Several lines of evidence suggest that components of the PBF transcriptional factor are also directly involved in the regulation of this group of genes. First, in addition to Ace2-binding sites most of the promoters also contain one or more copies of the PCB (GNAACR) and/or forkhead-binding (TGTTTA) sequences in different arrangements. Interestingly, in many cases these regulatory sequences are localized to nucleosome-depleted regions (NDRs), which are normally present immediately upstream of the transcription start site (TSS). It has been proposed that the precise organization of nucleosomes in promoters regulates the interaction between transcription factors and DNA,[35,44,45] suggesting that they can play a role in the regulation of this group of genes. Second, according to ChIP experiments, Fkh2 can be detected at the promoter region of several of these genes (*eng1*$^+$, *adg2*$^+$ and *adg3*$^+$), and Sep1 can also be immunoprecipitated associated with the promoter region of *eng1*$^+$ and *adg2*$^+$. Third, the expression of the Ace2-dependent genes was altered in *sep1*Δ, *mbx1*Δ,

*fkh2*Δ or *mbx1*Δ *fkh2*Δ mutants, although the effect of the deletion of each factor had different effects on individual genes, suggesting a different contribution of Fkh2, Sep1 and Mbx1 to the regulation of the expression of Ace2-dependent genes. Finally, although the expression of Ace2 is itself dependent on the PBF factor,[4,12,46] the effect on Ace2-dependent genes might be direct, since defects in expression were observed when the *ace2*$^+$ promoter was replaced with the regulated *nmt1*$^+$ promoter. Together, these results indicate that the PBF directly contributes to the regulation of these genes at the M-G1 transition.

It has previously been shown that expression of the *cdc15*$^+$ gene cluster requires the PBF transcription factor complex containing Sep1, Fkh2 and Mbx1,[12] with the two forkhead transcription factors playing opposing roles. The activator, Sep1, would only be bound to PCB promoters when the genes are expressed, and the repressor, Fkh2, appears to be bound when the genes are repressed.[19,24] However, the precise role of Fkh2 remains unclear. More recently, it has been proposed that this transcription factor would regulate the onset of mitotic transcription and the timing of mitotic entry, and that the primary function of Fkh2 could be the regulation of mitotic progression and the timing of transcription.[25] Our ChIP and expression analyses on synchronized cultures suggest that Ace2 and Fkh2 are also present at the promoters of Ace2-dependent genes, and that they are bound at different times of the cell cycle. Thus, Ace2 was found in the promoters of *eng1*$^+$, *agn1*$^+$ and *adg2*$^+$ when expression was high, while Fkh2 promoter occupancy occurred when the genes were repressed. Therefore, the regulation of Ace2-dependent genes shares some similarities with the Sep1-dependent genes, although the role of Fkh2 in regulating the expression of Ace2-dependent genes is not clear either, since no increase in *eng1*$^+$ expression was observed in synchronized cultures lacking Fkh2. Garg *et al.* (2015) have recently found by ChIP-seq that Sep1 only controls the transcription of a few genes and that a new regulator, Sak1, is apparently the main activator of mitotic gene expression.[29] These authors propose that Fkh2 could act as a pioneer factor to displace nucleosomes from regulatory regions in order to aid the assembly of an activating transcription complex.

It has been also shown that Mediator, a co-regulator of eukaryotic transcription that functions as a bridge between gene-specific regulators and RNA polymerase II,[47] is recruited to a large number of mitotic genes and regulates their transcription. This complex is present in the promoters of most Sep1-dependent genes and it has been proposed that Sep1 might be required for the recruitment of Mediator to target genes to ensure the correct regulation of periodic transcription.[48] Interestingly, this study also found that Mediator was recruited to the promoter of some the Ace2-dependent genes, such as *chf4*$^+$, *eng1*$^+$ or *adg2*$^+$, but not to *agn1*$^+$ or *adg3*$^+$. Since genes containing a higher number of

regulatory elements in their promoters (including the PCB and/or forkhead-binding sequences, Figure 2) correlate with those that recruit Mediator to the promoter, it is tempting to speculate that the presence of these regulatory elements in Ace2-dependent genes could be important for the correct regulation of periodic transcription.

One interesting conclusion from the observations presented here is that the regulation of Ace2-dependent genes is more complex than previously thought. Periodic expression of this group of genes requires the transcription factor Ace2,[9,13,49] but there are important differences in the regulation of the genes encoding the two main glucanases involved in cell separation, the endo-β1,3-glucananse Eng1 and the α1,3-glucananse Agn1. While the regulation of *eng1*$^+$ required Ace2 and Sep1, the expression of *agn1*$^+$ was dependent on Mbx1. We were unable to monitor the association of Mbx1 with the *agn1*$^+$ promoter by ChIP, as previously reported,[19] probably because tagged versions of Mbx1 are non-functional. However, the fact that the over-expression of *agn1*$^+$ complemented the separation defect of *mbx1*Δ mutants is a strong indication that Mbx1 regulates the expression of *agn1*$^+$. In *S. cerevisiae*, the MADS box transcription factor Mcm1 has an important regulatory function during cell cycle expression, controlling the expression of different group of genes at the M/G1 and G2/M transitions,[50,51] whereas the role of Mbx1 in *S. pombe* is less clear. Our results indicate that *agn1*$^+$ is under the control of Mbx1. These observations therefore indicate that the regulation of Ace2-dependent genes is heterogeneous, and that different components of the PBF might function differentially in each promoter. A question that arises is why cells use a different type of regulation for two genes that participate in the same biological process. One possible explanation is the observation that *agn1*$^+$ overexpression is lethal for the cells, whereas the overexpression of *eng1*$^+$ is not deleterious.[15-17] Since α-glucan is present in the septum region and surrounds the cell wall, where it plays a structural role and is essential for maintaining cell shape and viability,[52] it might be necessary for the cells to strictly regulate the moment when Agn1 is synthesized in order to avoid cell lysis. In contrast, Eng1 seems to be highly specific for the linear β-1,3 glucan of the primary septum,[53,54] not acting on other polymers of the cell wall, and therefore it might not be necessary for its expression to be tightly regulated.

MATERIALS AND METHODS

Yeast strains, growth conditions and genetic manipulations

Table 1 lists the yeast strains used in this work. Yeast cells were grown on YES medium or minimal medium (EMM) with the required supplements.[55] Yeast transformations were performed using standard procedures: the lithium acetate method or genetic crossing. For experiments using the *nmt1*$^+$ promoter, cells were grown to the logarithmic phase in EMM containing 15 μM thiamine, harvested, washed three times with EMM, and inoculated in

fresh medium without thiamine at an OD_{595}=0.025. Synchronization of strains carrying the thermosensitive *cdc25-22* mutation was achieved by growing the cells at the permissive temperature (25°C) to early log phase (OD_{595}=0.35) and then shifting the cultures to 37°C for 4 h. Cells were released from arrest by transfer to 25°C, and samples were taken every 10 or 20 min. Synchrony was monitored by estimation of the percentage of binucleate and septated cells under the microscope.

Construction of plasmids and strains

Plasmid pMAN4, containing the *eng1*$^+$ promoter was constructed by PCR amplification with oligonucleotides that generated *Sph*I and *Bam*HI sites at the ends and then cloning the amplified fragment into the corresponding sites of vector pSPE357, which contains the *Escherichia coli* l*acZ* gene and the *ura4*$^+$ marker.[56] Deletion of the first (Δ1), the second (Δ2), or both (Δ1Δ2) copies of the CCAGCC sequence was achieved by recombinant PCR, generating the desired deletions as *Sph*I-*Bam*HI fragments, which were cloned into plasmid pSPE-357 to yield plasmids pMAN5, pMAN6 and pMAN7 respectively.

ace2$^+$ null mutants were obtained by replacing the *ace2*$^+$ coding region with the *kan*^{r} cassette (which confers resistance to the antibiotic G418) or the *ura4*$^+$ gen by recombinant PCR as described.[57] For this purpose, DNA fragments of 300–500 bp of the 5´ and 3´ flanking regions of *ace2*$^+$ were PCR-amplified using specific oligonucleotide pairs. The resulting fragments were then fused by recombinant PCR to the corresponding cassette.

Strains carrying the *ace2*$^+$ gene tagged with HA were constructed by a PCR-mediated strategy using the *3HA-kanMX6* module for C-terminal tagging.[58] Strains with *ace2*$^+$ under the control of the *nmt1*$^+$ promoter at its chromosomal locus were constructed using the *kanMX6-P41nmt1* [58] or the *natMX6-P41nmt1-GFP* (which confers resistance to the antibiotic nourseothricin) modules for inducible expression.[59] In all cases, the tagging and the deletion cassettes were obtained by PCR with oligonucleotides containing approximately 100 base pairs of flanking sequences homologous to the target sequence.

Strains bearing P*eng1*-Δ1Δ2 or P*eng1-4m* alleles were constructed using an *in vivo* site-directed mutagenesis system to create unmarked mutant alleles, in which the target locus was initially marked with the *ura4*$^+$ gene, after which the marker was replaced with the mutated DNA by counterselection on medium containing 5-fluoroorotic acid (5-FOA).[60] For this, a DNA fragment for replacing the *eng1*$^+$ promoter with the *ura4*$^+$ cassette was generated by PCR amplification of plasmid pFA6a-ura4 [58] with specific oligonucleotides, and this PCR product was used to transform strains YMAT14 and YMAT15 to the Ura$^+$, yielding strains YMAT40 and YMAT41, respectively. A PCR product carrying the *Peng1*-Δ1Δ2 allele obtained by PCR using pMAN7 as template was used to transform strains YMAT40 and YMAT41, selecting 5FOA^R colonies that yielded strains YMAT84 and

YMAT85, respectively. A similar approach was used to insert the mutations in the three forkhead (GTAAACA to GTAGCG) and the PCB sites of the *eng1*$^{+}$ promoter (P*eng1-4m* allele). Mutations of these four sites were achieved by successive rounds of PCR, and the final product was used to transform strains YMAT40 and YMAT41. Then, 5FOA^{R} colonies were selected, yielding strains YMAT42 and YMAT43, respectively. Strains YMAT69 and YMAT70 were obtained by crossing YMAT16 and YMAT42. In all cases, proper integration of the corresponding cassettes in the *S. pombe* genome was confirmed by PCR. The oligonucleotide sequences used for strain constructions are available upon request.

Microscopy

Samples were observed on a Nikon Eclipse i90 microscope equipped with a Hamamatsu Orca-ER camera and controlled by MetaMorph (Molecular Devices Corporation). Visualization of septa was accomplished by staining yeast cells with aniline blue. For synchrony analysis, cells fixed in 70% ethanol were stained simultaneously with DAPI (4,6-α-diamidino-2-phenylindole) and aniline blue.

Protein extracts and β-galactosidase assay

β-galactosidase activity was determined using ONPG (o-nitrophenyl-β-D-galactopyranoside) as substrate. Yeast cells were grown to the logarithmic phase, harvested by centrifugation, washed, and suspended in lysis buffer (100 mM Tris-HCl, pH 8.0, 20% glycerol, 1 mM β-mercaptoethanol, 40 mM PMSF). After breaking the cells, lysates were cleared by centrifugation, and 50 µl of the supernatant was used in a 1-ml ONPG assay as described.[61] Specific enzyme activity was calculated in Miller units.

Electromobility Bandshift Assays (EMSA)

Whole cell extracts were generated from cells as described and gel retardation analysis was performed with an *eng1*$^{+}$ promoter obtained by PCR amplification with specific oligonucleotides in the presence of α-^{32}P dCTP, as previously described.[12]

Northern blot analyses

Cells (10^{9}) were collected at different time intervals after release from the restrictive temperature (37°C), and total RNA was prepared as previously described.[15] RNAs (12.5 µg) were transferred to Hybond membranes and probed with ^{32}P-labeled probes corresponding to *eng1*$^{+}$ and *agn1*$^{+}$ and *act1*$^{+}$ obtained by PCR with specific oligonucleotides. Signals were normalized using *act1*$^{+}$ transcript level.

Gene expression analysis by RT-qPCR

To determine the expression of genes by quantitative RT-PCR, 2.0 X 10^8 cells were collected by centrifugation and used for total RNA extraction using the TRIZOL method (Invitrogen), according to the manufacturer's instructions. cDNA synthesis was carried out with the SuperScript II First-Strand Synthesis System (Invitrogen), using 3 µg of RNA previously treated with DNAse I (Invitrogen). 1 µl of cDNA was used for the quantitative reactions in an Applied Biosystems 7300 Real-Time PCR System. The SYBR *Premix Ex Taq* (TaKaRa) reagent was used with primer concentrations of 0.2 µM. Serial dilutions of wild-type *S. pombe* genomic DNA (1/10, 1/100, 1/1000, 1/10000, 1/100000) were used to generate a standard curve for each reaction. The reaction conditions were as follows: 95°C for 45 s and 40 cycles of 95°C for 5 s and 60°C for 31 s, followed by a dissociation step at 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. All PCR reactions were normalized to *his3*$^+$ or *act1*$^+$ transcription data. The experiments were performed at least twice using cDNA from different biological replicates with two technical replicates for each sample. The "Mean Normalized Expression" was calculated according to Simon. [62]

ChIP-qPCR

Cells (10^9) bearing an HA or a c-myc tag from mid-log-stage cultures were collected and used for ChIP assays according to Robyr and Grunstein [63], with the following modifications: cells were lysed using a FastPrep-24 bead beater at a speed setting of 4.5 for 40 s followed by 5.0 2X 40 s, at 4ºC. The crude lysate was sonicated on ice using a Diagenode Bioruptor Sonicator (at settings: "high"; 30 s ON, 30 s OFF) for 30 min (15 min cumulative sonication time). Cell debris was centrifuged for 30 min at 13000 *g* at 4ºC. A total of 15 µg of antibody specific for anti-HA (12CA5, Roche) or c-myc (9E10, Santa Cruz) was applied in a 500-µl volume of chromatin. The experiments were performed twice with 2 immunoprecipitation repeats in each experiment. Cells from the untagged control strain (OL264) were also collected, using the same experimental conditions. A 1-µl volume of ChIP DNA was used for qPCR. The reaction conditions were performed as described above.

For ChIP experiments along the cell cycle, cells were synchronized in *cdc25-22* block-release experiments, and collected every 20 min after release from the restrictive temperature (37°C). Samples for each time-point were divided into two aliquots, one (10^9 cells) processed for ChIP and the other ($2x10^8$ cells) processed for mRNA expression analyses. Cells from the untagged control strain were also collected using the same experimental setting.

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FIGURE LEGENDS

Figure 1. Ace2 regulates the expression of its target genes by periodically binding the CCAGCC sequence. A) Schematic representation of the position of the two copies of the CCAGCC sequence in the *eng1*$^+$ promoter and constructs generated. The different deletions were cloned upstream of the *E. coli lacZ* gene. The arrow indicates the orientation of the CCAGCC sequence. The graph shows the results of the β-galactosidase activity assay in the wild-type strain (PN1870) carrying the wild-type *eng1*$^+$ promoter (WT; pMAN4), *eng1*-Δ1 (pMAN5), *eng1*-Δ2 (pMAN6), *eng1*-Δ1Δ2 (pMAN7) or empty vector and in the *ace2*Δ mutant (OL163) transformed with the wild-type *eng1*$^+$ promoter (WT; pMAN4). The data are means (±SD) of 2 independent experiments and are normalized to the wild-type strain. **B)** Chromatin immunoprecipitation of Ace2-HA in a strain carrying the wild-type *eng1*$^+$ promoter (WT; YMAT15) and in a strain carrying the deletion of the putative Ace2-binding sites in the *eng1*$^+$ promoter (*eng1*-Δ1Δ2; YMAT85). Data are shown relative to the background binding in untagged control cells (OL264). The binding of Ace2 to the *his3*$^+$ promoter was used as a negative control to normalize the data. The columns represent the mean of two independent biological repeats, indicated by dots. Each dot is the mean of at least two technical replicates. **C)** *eng1*$^+$ expression level in the wild-type strain (WT, YMAT15) and in the *eng1*-Δ1Δ2 (YMAT85) and *ace2*Δ (YMAT71) mutants determined by quantitative RT-PCR and normalized using *his3*$^+$ expression. The columns represent the mean of two independent biological repeats, indicated by dots. Each dot is the mean of at least two technical replicates. **D)** Expression of *eng1*$^+$ and *agn1*$^+$ in synchronized cells. Data were normalized using *his3*$^+$ expression. The results shown are representative of the results obtained in two different experiments. **E)** Ace2 binding to the promoters of *eng1*$^+$ and *eng1*$^+$ along the cell cycle. A *cdc25-22 ace2-HA* strain (YMAT15) was synchronized by arrest-release and samples were taken at the indicated times (minutes) after the release for mRNA purification (D) or chromatin immunoprecipitation (E) using anti-HA antibodies. The anaphase and septation indices are indicated in E. Data are shown relative to the background binding in untagged control cells (OL264). The binding of Ace2-HA to the *his3*$^+$ promoter was used as a negative control to normalize the data. The results shown are representative of the results obtained in two different experiments.

Figure 2. Diverse binding sites are present in the promoters of Ace2-dependent genes. Schematic representation of the promoter region of Ace2-dependent genes. Blue rectangles indicate the position of the CCAGCC sequence; green rectangles correspond to TGTTTA motifs, and red rectangles mark GNAACR sequences. The position of the rectangles indicates their orientation: direct if it is above the line and inverted if it is below. NDR regions in the promoters are indicated by yellow boxes. Genes were ordered

according to the abundance of these sites within NDR. The asterisks indicate the sites mutated in this study (TGTTTA to CGGCTA and GNAACR to GNGCCR).

Figure 3. PBF components bind to Ace2-target promoters *in vitro* and *in vivo.* A) Gel retardation assay with the *eng1*$^+$ promoter. A labeled DNA fragment corresponding to the region marked with a rectangle in the schematic representation to the left was incubated with protein extracts from the wild-type strain (PN1870, WT) and the *ace2*Δ (OL163), *mbx1*Δ (GG503), *fkh2*Δ (GG523), *mbx1*Δ *fkh2*Δ (GG552), *ace2*Δ *fkh2*Δ (YMAN91), *ace2*Δ *mbx1*Δ (YMAN92) and *ace2*Δ *mbx1*Δ *fkh2*Δ (YMAN106) mutants. The probe without protein extract (F) is also shown. **B)** Chromatin immunoprecipitation of Ace2-HA, Fkh2-HA and Sep1-myc in *ace2-HA* (YMAT15), *fkh2-HA* (YMAT14) or *sep1-myc* (YMAT16) strains. Data are shown relative to the background binding in untagged control cells (OL264). Binding of the three tagged proteins to *his3*$^+$ promoter was used as a negative control to normalize the data. **C)** *eng1*$^+$ promoter scanning. Schematic representation of the intergenic region that separates *slp1*$^+$ and *eng1*$^+$, in which the binding sites for Ace2 are indicated with light gray boxes and those for forkhead transcription factors with dark gray. The oligonucleotide pairs used for ChIP experiments are also indicated. Below, quantitative results of the ChIP experiments with Ace2-HA or Fkh2-HA. Data are shown relative to the background binding in untagged control cells (OL264). The binding of Ace2-HA and Fkh2-HA to the *his3*$^+$ promoter was used as a negative control to normalize the data. For B and C, the columns represent the mean of two independent biological repeats, indicated by dots. Each dot represents the mean of at least two technical replicates.

Figure 4. Regulation of *eng1*$^+$ and *agn1*$^+$ requires different factors. A) Expression of *ace2*$^+$ and the Ace2-target genes *eng1*$^+$ and *agn1*$^+$ measured by quantitative RT-PCR in the wild-type strain (OL432) and the *ace2*Δ (YMAN30), *sep1*Δ (A131), *fkh2*Δ (GG523), *mbx1*Δ (GG503) and *mbx1*Δ *fkh2*Δ (GG552) mutants. *his3*$^+$ expression was used for normalization. **B)** Expression of *eng1*$^+$ and *agn1*$^+$ measured by quantitative RT-PCR in strains $P_{nmt}$$^+$-*ace2*$^+$ (YMAT59), *mbx1*Δ P_{nmt1}-*ace2*$^+$ (YMAT61), *sep1*Δ P_{nmt1}-*ace2*$^+$ (YMAT62), and *fkh2*Δ P_{nmt1}-*ace2*$^+$ (YMAT60). The graph represents the quantification of the expression of each gene with respect to strain P_{nmt1}-*ace2*. *act1*$^+$ expression was used for normalization. In both panels, the columns represent the mean of two independent biological repeats, indicated by dots. Each dot represents the mean of at least two technical replicates.

Figure 5. Fkh2 binds to the promoters of Ace2-dependent genes at different moments of the cell cycle. A) Chromatin immunoprecipitation of Fkh2-HA in the *cdc25-22*

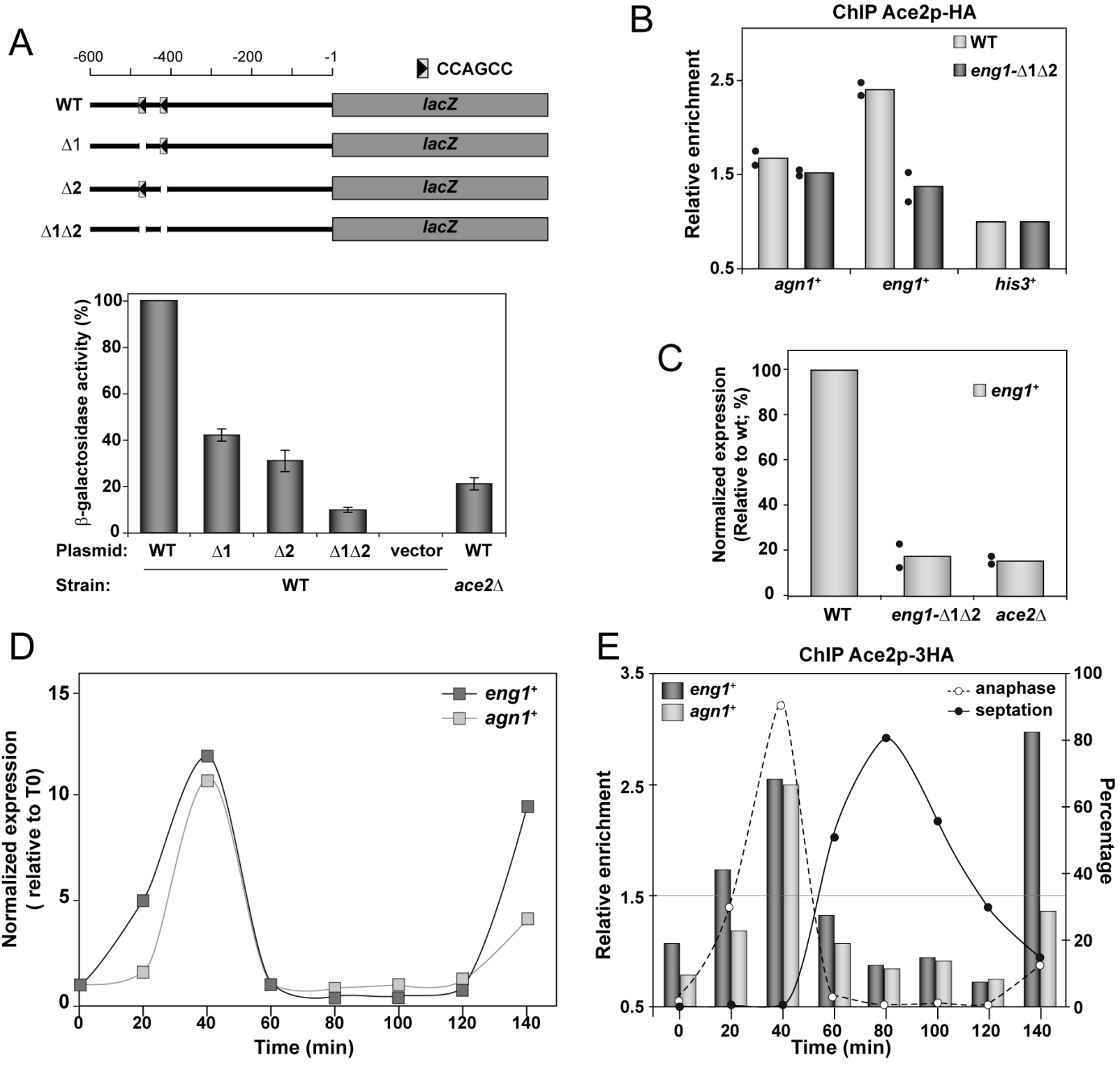
fkh2-HA strain (YMAT14) (left), and expression of *eng1*$^+$ and *agn1*$^+$ (right) measured by quantitative RT-PCR along the cell cycle and normalized using *his3*$^+$ expression. Synchrony was induced by arrest-release and samples were taken at the indicated times (minutes) after the release for chromatin immunoprecipitation (left) using anti-HA antibodies or for mRNA purification (right). The anaphase and septation index is indicated in the left graph. Data are shown relative to the background binding in untagged control cells (OL264). Binding of Fkh2-HA to the *his3*$^+$ promoter was used as a negative control to normalize the data. **B)** Expression of *eng1*$^+$ determined by quantitative RT-PCR in strains *nda3-KM311* P*nmt1*$^+$*-ace2*$^+$ (YMAT17), and *nda3-KM311 fkh2*Δ P*nmt1*$^+$*-ace2*$^+$ (YMAT94) along the cell cycle. Cells were arrested in early mitosis by incubation at 18ºC and samples were taken at the indicated times (minutes) after release for mRNA purification. Expression data were normalized using *his3*$^+$ expression. **C)** Chromatin immunoprecipitation of Fkh2-HA and Sep1-myc in strains carrying mutations in the three forkhead binding sites and an adjacent PCB site in the *eng1*$^+$ promoter (P*eng1-4m* allele) (strains YMAT43 and YMAT70, respectively). Data are shown relative to the binding in wild-type cells (YMAT14 and YMAT69, respectively). The binding of Fkh2-HA and Sep1-myc to the *his3*$^+$ promoter was used as a negative control to normalize the data. The columns represent the mean of two independent biological repeats, indicated by dots. Each dot represents the mean of at least two technical replicates. **D)** Expression of *eng1*$^+$ and *agn1*$^+$ during the cell cycle. Synchrony was induced by arrest-release of *cdc25-22* (YMAT14) or *cdc25-22* P*eng1-4m* (YMAT43) mutants, and samples were taken at the indicated times (minutes) after release for RNA extraction. Expression was measured by quantitative RT-PCR and normalized using *his3*$^+$ expression. For the time course experiments (A, B and D), the results shown are representative of the results obtained in two different experiments.

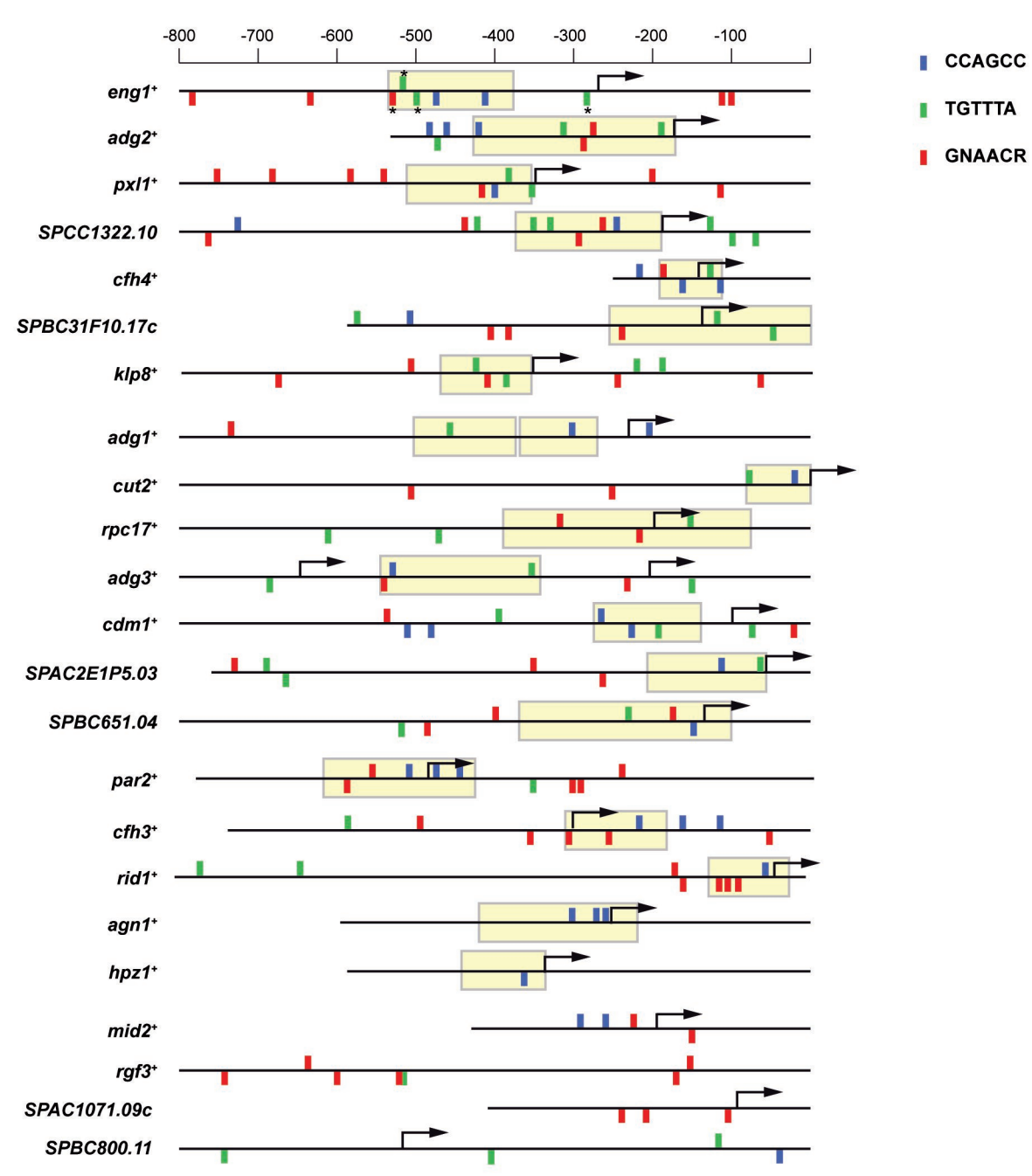
Figure 6. Mbx1 controls *agn1*$^+$ expression. A) Expression of *eng1*$^+$ and *agn1*$^+$ during the cell cycle. Synchrony was induced by arrest-release of *cdc25-22* (OL264) or *cdc25-22 mbx1*Δ (GG549) mutants, and samples were taken at the indicated times (minutes) after release for RNA extraction. RNA blots were probed with specific probes for *eng1*$^+$ and *agn1*$^+$, using *act1*$^+$ as a loading control. The percentage of septation at each time-point is indicated below, and was determined by counting the percentage of cells with a septum after calcofluor staining. The graph represents the quantification of the expression of each gene with respect to the wild-type (wt, value 1). **B)** Overexpression of *agn1*$^+$ complements the separation defects of *mbx1*Δ mutants. The wild-type (WT; OL432) and the *agn1*Δ (YSAB156), *mbx1*Δ (GG503) and *mbx1*Δ carrying P*nmt1*$^+$*-agn1*$^+$ (YMAT91) mutants were grown in EMM5S medium without thiamine for 17 hours before staining the cells with aniline blue. Images show fields and details of separating cells for each strain. The graph to

the right indicates the percentage of cells with a septum that have a wild-type or a V-shaped phenotype in each strain (n=350).

Table 1. Yeast strains used in this study.

Strain	Genotype	Origin
A131	*h$^+$ ura4-D18 leu1-32 sep1::ura4$^+$*	M. Sipiczki
GG503	*h$^-$ ura4-D18 ade6-M210 leu1-32 his7-866 mbx1::kanR*	C. McInerny
GG523	*h$^+$ ura4-D18 leu1-32 fkh2::kanR*	C. McInerny
GG549	*h$^-$ cdc25-22 leu1-32 ura4-D18 mbx1::kanR*	C. McInerny
GG552	*h$^+$ ura4-D18 leu1-32 his7-866 fkh2::kanR mbx1::kanR*	C. McInerny
OL163	*h$^-$ ura4-D18 ace2::kanR*	Lab stock
OL264	*h$^-$ cdc25-22 ura4-D18*	Lab stock
OL1143	*h$^+$ nda3-KM311 leu1-32*	S. Moreno
OL432	*h$^-$ ura4-D18 leu1-32*	Lab stock
PN1870	*h$^+$ ura4-D18 leu1-32*	P. Pérez
YMAN106	*h$^+$ ura4-D18 leu1-32 his7-866 fkh2::kanR mbx1::kanR ace2::ura4$^+$*	This study
YMAN30	*h$^-$ ura4-D18 leu1-32 ace2::kanR*	Lab stock
YMAN91	*h$^+$ ura4-D18 leu1-32 fkh2::kanR ace2::ura4$^+$*	This study
YMAN92	*h$^-$ ura4-D18 ade6-M210 leu1-32 his7-866 mbx1::kanR ace2::ura4$^+$*	This study
YMAT14	*h$^-$ cdc25-22 ura4-D18 fkh2-3HA-kanR*	This study
YMAT15	*h$^+$ cdc25-22 ura4-D18 ace2-3HA-kanR*	This study
YMAT16	*h$^-$ cdc25-22 leu1-32 sep1-13myc-kanR*	This study
YMAT17	*h? nda3-KM311 leu1-32? kanMX6-P41nmt1-GFP-ace2$^+$*	This study
YMAT40	*h$^+$ cdc25-22 ura4-D18 ace2-3HA-kanR Peng1::ura4$^+$*	This study
YMAT41	*h$^-$ cdc25-22 ura4-D18 fkh2-3HA-kanR Peng1::ura4$^+$*	This study
YMAT42	*h$^+$ cdc25-22 ura4-D18 ace2-3HA-kanR Peng1-4m*	This study
YMAT43	*h$^-$ cdc25-22 ura4-D18 fkh2-3HA-kanR Peng1-4m*	This study
YMAT59	*h$^-$ ura4-D18, leu1-32 natMX6-P41nmt1-ace2$^+$*	This study
YMAT60	*h$^+$ ura4-D18 leu1-32 fkh2::kanR natMX6-P41nmt1-ace2$^+$*	This study
YMAT61	*h$^-$ ura4-D18 ade6-M210 leu1-32 his7-866 mbx1::kanR natMX6-P41nmt1-ace2$^+$*	This study
YMAT62	*h? ura4-D18 leu1-32 sep1::ura4$^+$ natMX6-P41nmt1-ace2$^+$*	This study
YMAT69	*h? cdc25-22 ura4-D18? leu1-32? ace2-3HA-kanR sep1-13myc-kanR*	This study
YMAT70	*h? cdc25-22 ura4-D18? leu1-32? ace2-3HA-kanR sep1-13myc-kanR Peng1-4m*	This study
YMAT71	*h? cdc25-22 ura4-D18 leu1-32 ace2::kanR*	This study
YMAT84	*h$^-$ cdc25-22 ura4-D18 fkh2-3HA-kanR Peng1-*Δ1Δ2	This study
YMAT85	*h$^+$ cdc25-22 ura4-D18 ace2-3HA-kanR Peng1-*Δ1Δ2	This study
YMAT91	*h$^-$ ura4-D18 ade6-M210 leu1-32 his7-866 mbx1::kanR* (pREP3X-*agn1$^+$*)	This study
YMAT94	*h? nda3-KM311 leu1-32? fkh2::natMX6 kanMX6-P41nmt1-GFP-ace2$^+$*	This study
YSAB156	*h$^+$ ura4-D18 leu1-32 ade6-M216 agn1::kanR*	This study


A
-600
-400
-200
-1
CCAGCC
WT
lacZ
Δ1
lacZ
Δ2
lacZ
Δ1Δ2
lacZ
β-galactosidase activity (%)
100
80
60
40
20
0
Plasmid:
WT
Δ1
Δ2
Δ1Δ2
vector
WT
Strain:
WT
ace2Δ
B
ChIP Ace2p-HA
WT
eng1-Δ1Δ2
Relative enrichment
2.5
1.5
0.5
agn1+
eng1+
his3+
C
Normalized expression
(Relative to wt; %)
100
80
60
40
20
0
eng1+
WT
eng1-Δ1Δ2
ace2Δ
D
eng1+
agn1+
Normalized expression
(relative to T0)
15
10
5
0
0
20
40
60
80
100
120
140
Time (min)
E
ChIP Ace2p-3HA
eng1+
agn1+
anaphase
septation
Relative enrichment
3.5
2.5
1.5
0.5
Percentage
100
80
60
40
20
0
0
20
40
60
80
100
120
140
Time (min)


-800
-700
-600
-500
-400
-300
-200
-100
CCAGCC
TGTTTA
GNAACR
eng1+
adg2+
pxl1+
SPCC1322.10
cfh4+
SPBC31F10.17c
klp8+
adg1+
cut2+
rpc17+
adg3+
cdm1+
SPAC2E1P5.03
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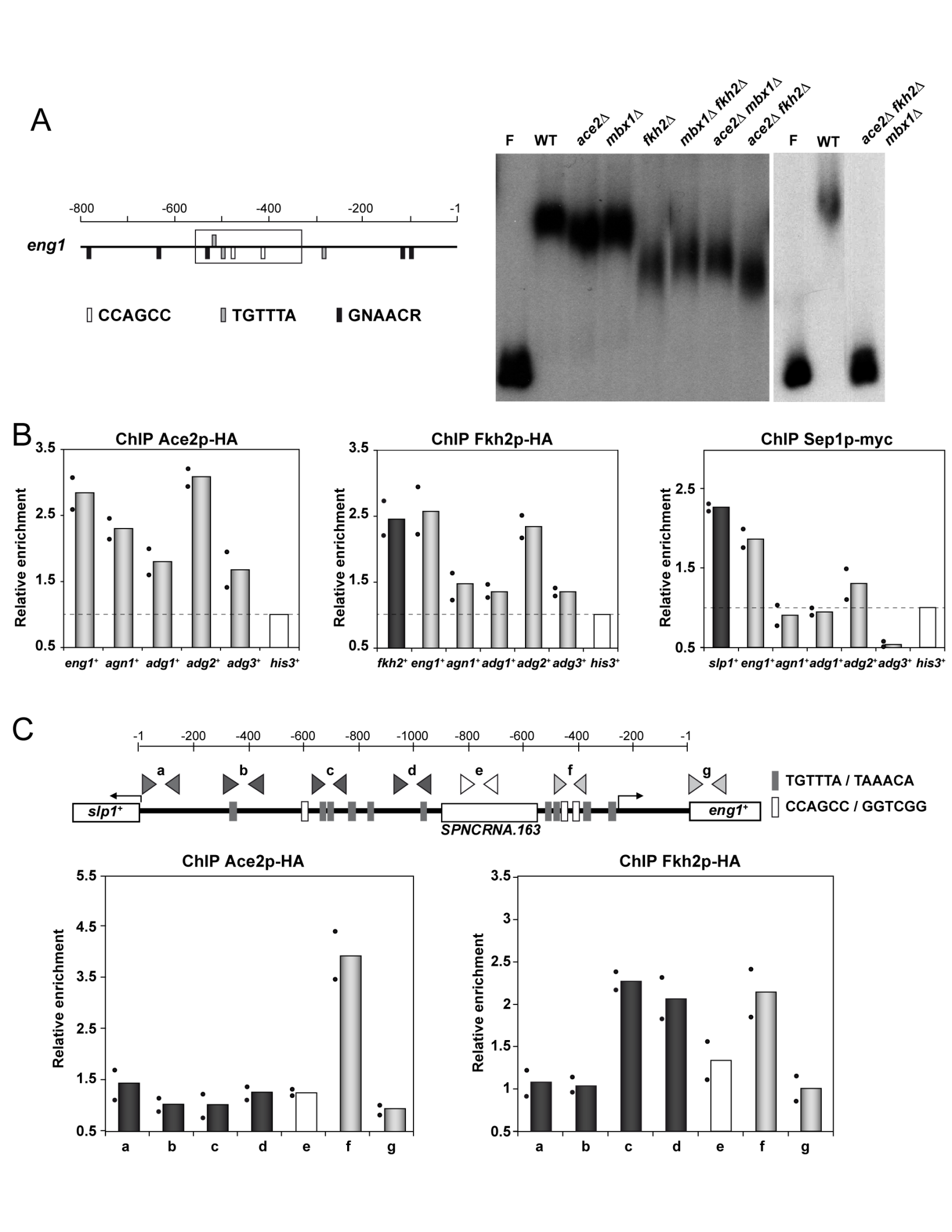
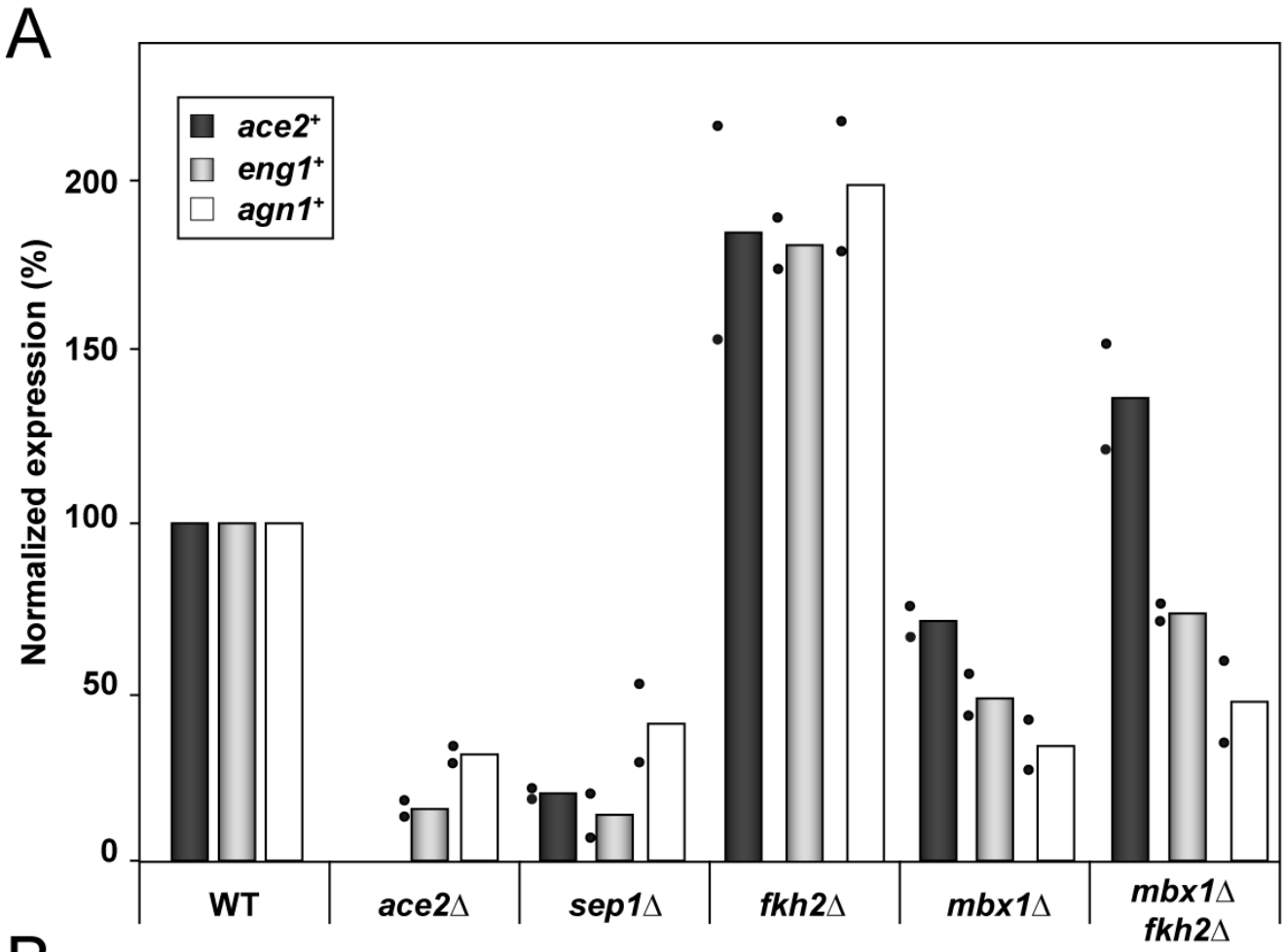
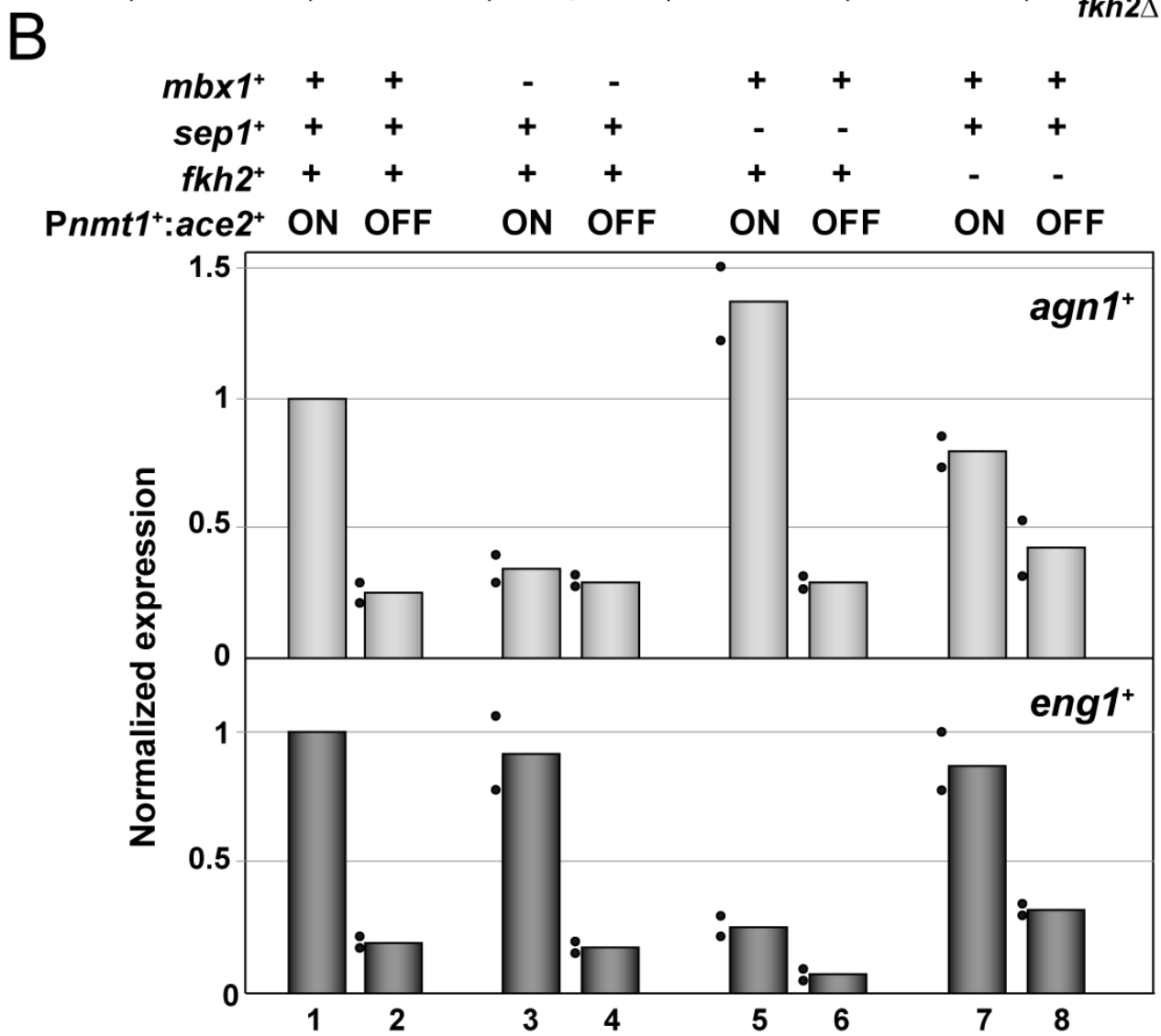
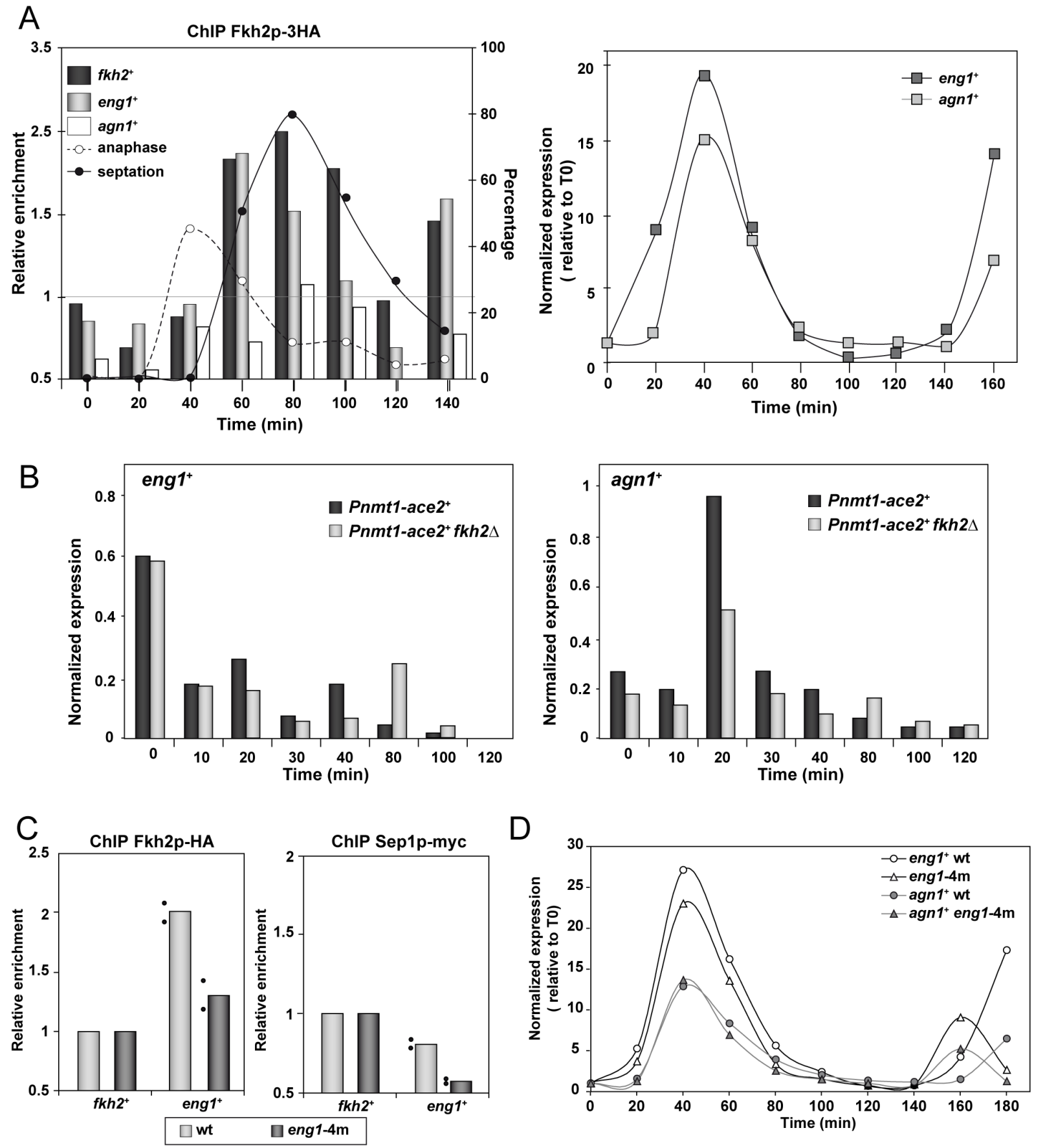
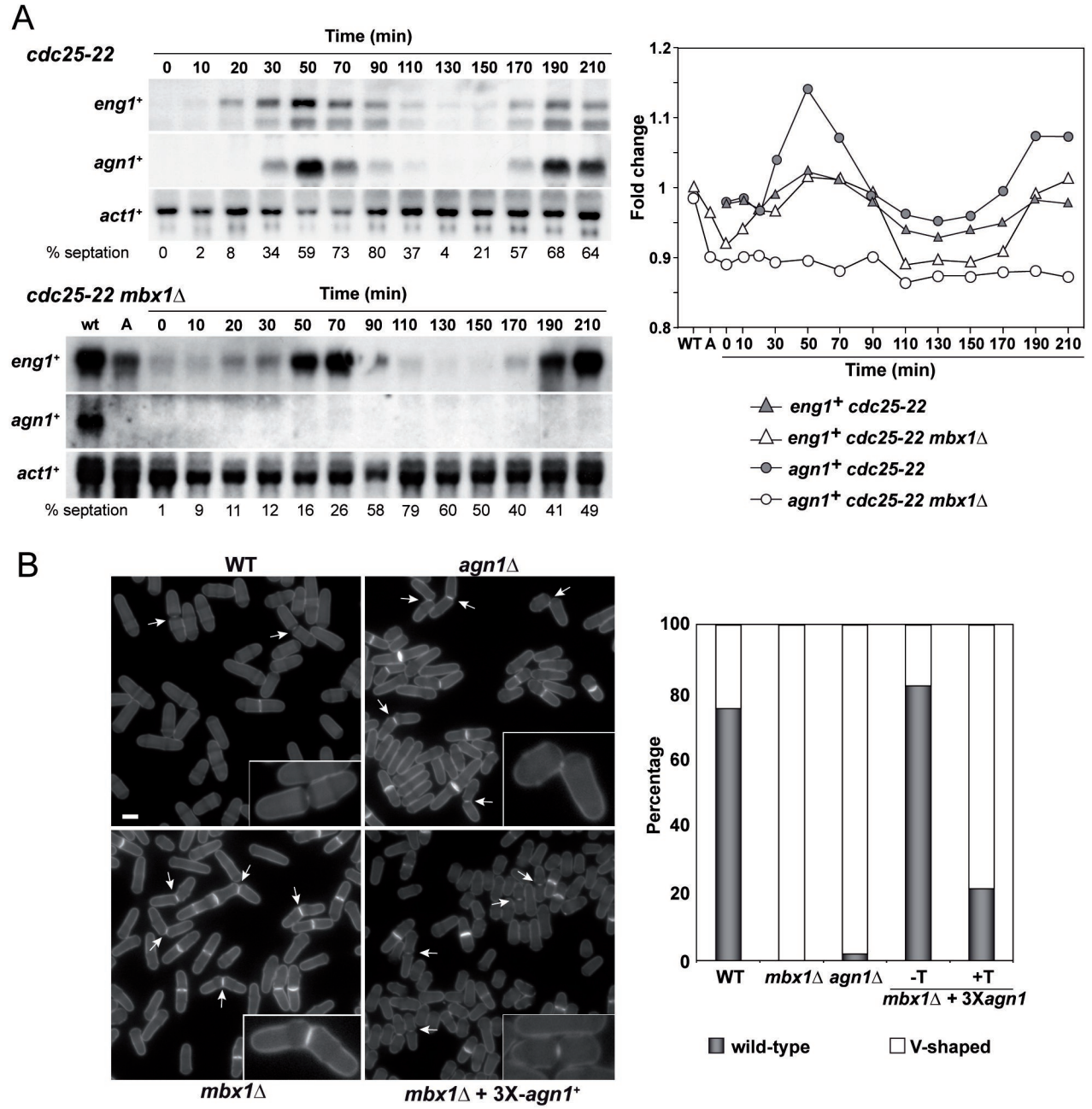
par2+
cfh3+
rid1+
agn1+
hpz1+
mid2+
rgf3+
SPAC1071.09c
SPBC800.11


A
F
WT
ace2Δ
mbx1Δ
fkh2Δ
mbx1Δ fkh2Δ
ace2Δ mbx1Δ
ace2Δ fkh2Δ
F
WT
ace2Δ fkh2Δ
mbx1Δ
-800
-600
-400
-200
-1
eng1
CCAGCC
TGTTTA
GNAACR
B
ChIP Ace2p-HA
Relative enrichment
3.5
2.5
1.5
0.5
eng1+ agn1+ adg1+ adg2+ adg3+ his3+
ChIP Fkh2p-HA
Relative enrichment
3.5
2.5
1.5
0.5
fkh2+ eng1+ agn1+ adg1+ adg2+ adg3+ his3+
ChIP Sep1p-myc
Relative enrichment
2.5
1.5
0.5
slp1+ eng1+ agn1+ adg1+ adg2+ adg3+ his3+
C
-1
-200
-400
-600
-800
-1000
-800
-600
-400
-200
-1
a
b
c
d
e
f
g
slp1+
eng1+
SPNCRNA.163
TGTTTA / TAAACA
CCAGCC / GGTCGG
ChIP Ace2p-HA
Relative enrichment
5.5
4.5
3.5
2.5
1.5
0.5
a b c d e f g
ChIP Fkh2p-HA
Relative enrichment
3.5
3
2.5
2
1.5
1
0.5
a b c d e f g


A
ace2+
eng1+
agn1+
Normalized expression (%)
200
150
100
50
0
WT
ace2Δ
sep1Δ
fkh2Δ
mbx1Δ
mbx1Δ fkh2Δ


B
mbx1+ + + - - + + + +
sep1+ + + + + - - + +
fkh2+ + + + + + + - -
Pnmt1+:ace2+ ON OFF ON OFF ON OFF ON OFF
Normalized expression
agn1+
eng1+
1.5
1
0.5
0
1 2 3 4 5 6 7 8


A
ChIP Fkh2p-3HA
fkh2+
eng1+
agn1+
anaphase
septation
Relative enrichment
Percentage
Time (min)
Normalized expression
(relative to T0)
eng1+
agn1+
Time (min)
B
eng1+
Pnmt1-ace2+
Pnmt1-ace2+ fkh2Δ
Normalized expression
Time (min)
agn1+
Pnmt1-ace2+
Pnmt1-ace2+ fkh2Δ
Normalized expression
Time (min)
C
ChIP Fkh2p-HA
Relative enrichment
fkh2+
eng1+
ChIP Sep1p-myc
Relative enrichment
fkh2+
eng1+
wt
eng1-4m
D
Normalized expression
(relative to T0)
eng1+ wt
eng1-4m
agn1+ wt
agn1+ eng1-4m
Time (min)


A
cdc25-22
Time (min)
0 10 20 30 50 70 90 110 130 150 170 190 210
eng1+
agn1+
act1+
% septation 0 2 8 34 59 73 80 37 4 21 57 68 64
cdc25-22 mbx1Δ
Time (min)
wt A 0 10 20 30 50 70 90 110 130 150 170 190 210
eng1+
agn1+
act1+
% septation 1 9 11 12 16 26 58 79 60 50 40 41 49
Fold change
1.2
1.1
1
0.9
0.8
WT A 0 10 30 50 70 90 110 130 150 170 190 210
Time (min)
eng1+ cdc25-22
eng1+ cdc25-22 mbx1Δ
agn1+ cdc25-22
agn1+ cdc25-22 mbx1Δ
B
WT
agn1Δ
mbx1Δ
mbx1Δ + 3X-agn1+
Percentage
100
80
60
40
20
0
WT
mbx1Δ
agn1Δ
-T
+T
mbx1Δ + 3Xagn1
wild-type
V-shaped